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14. ABSTRACT

The goal of this research was to investigate efficacy and mechanisms of H5BVIFN-β, a novel immunotherapeutic agent, against prostate cancer in animal models. The objective in year 3 was to investigate mechanisms by which H5BVIFN-β inhibits tumor growth. We found that the therapeutic effects of H5BVIFN-β were significantly reduced in macrophage-compromised mice and in mice deficient in inducible nitric oxide synthase. We found that intratumoral injection of H5BVIFN-β reduced microvessel density and downregulated expression of several angiogenic molecules, including transforming growth factor-β1, vascular endothelial cell growth factor, and platelet-derived growth factor. Expression of antiapoptotic molecule endothelin-1 was also downregulated. Our data suggest that H5BVIFN-β therapy suppressed tumor growth by downregulating expression of angiogenic molecules and, hence, tumor angiogenesis. This therapy should be beneficial to patient with localized prostate cancer.

15. SUBJECT TERMS
Prostate Cancer

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INTRODUCTION

The specific aims of this research are to be investigating efficacy of our novel immunotherapy system in the treatment of occult prostate cancer and mechanisms by which this therapy suppresses tumor growth. This is the final report of the entire research period.

BODY

1. Year 3 progress report

The tasks for years 1 and 2 were to determine the efficacy of our novel therapy in prostate cancer model. We found that our baculovirus-based interferon-beta system (H5BVIFN-β) was able to inhibit tumor growth (Year 1 report, Figs. 1 and 2; Year 2 report, Figs. 1,2, and 4) and prolong the survival of tumor-bearing mice (Year 1 Report, Fig. 3 and Year 2 report, Fig. 4). However, it fails to mount immune reaction to eradicate primary tumors and to confer systemic immune protection in mice receiving the therapy. Further analysis revealed that H5BVIFN-β failed to enhance the expression of immune-stimulatory cytokines necessary for the induction of tumor-specific immunity (Year 2 report, Fig. 5). These data suggest that inhibition of tumor growth by H5BVIFN-β was mediated by some local mechanisms.

In the year 3 of this project, with the approval from DOD-PCRP, we focused on research on local mechanisms that potentially mediates therapeutic effects of H5BVIFN-β in the orthotopic model of mouse prostate cancer and more specifically effects of the therapy on tumor angiogenesis. Our previous studies showed that macrophages are key component in suppressing tumor growth induced by an adenoviral vector system, we focused our study on the role of macrophage and inducible nitric oxide synthase (iNOS) in H5BVIFN-β therapy against TRAMP tumors.

1. Comparison of therapeutic effects of H5BVIFN-β in syngeneic mice and mice deficient in iNOS. TRAMP-C2RE3 cells (10^5/mouse) were injected into the prostate of male C57BL/6 or C57BL/6-iNOS knockout mice. Seven to 10 days after the orthotopic implantation, mice were intralesionally injected with 150 ug/injection (3 times a week

during the entire experiment period) of antibodies against Mac-1 and Mac-2 antigens that are selectively expressed on macrophages. After the second antibody injection, tumors were injected with PBS or 2 units of H5BVIFN-β. The experiments were terminated 3 weeks after the therapies. The prostate tumors were removed and weighed. As shown in Fig. 1, H5BVIFN-β therapy significantly inhibited growth of TRAMP tumors the wildtype C57BL/6 mice and therapeutic effects were reversed by administration of the antibodies against Mac-1 and Mac-2 antigens (Fig. 1). In contrast, H5BVIFN-β had no significant effects on TRAMP tumor growth in C57BL/6-iNOS KO mice that are have iNOS gene deletion. These data conclude that macrophages and iNOS expressed in macrophages are critical in suppressing TRAMP tumor growth in syngeneic mice.

2. Macrophage infiltration and iNOS expression in TRAMP tumors. We therefore investigated macrophage infiltration in the tumors. TRAMP cells were injected into C57BL/6 mice and treated with anti-Mac-1 and Mac-2 antibodies as well as H5BVIFN-β as detailed above. On day 7 after the therapy injection of H5BVIFN-β, tumors were sampled for immunohistochemical staining. As shown in Fig. 2, TRAMP-tumors were densely infiltrated by macrophages as revealed by immunostaining with macrophage-specific F4/80 antibody. Macrophage infiltration was significantly enhanced by intratumoral injection of H5BVIFN-β (Fig. 2). The administration of the antibodies against Mac-1 and Mac-2 antigen reduced the density of macrophage infiltration in tumors, due possibly to depletion of macrophages by the antibody (Fig.2). Immunostaining with antibody-specific to murine iNOS show that iNOS expression was not expressed in tumors injected with PBS but was found in tumors treated with H5BVIFN-β (Fig. 2). The iNOS expression was significantly reduced in tumors from mice treated with the antibodies against Mac-1 and Mac-2 antigens, indicating that iNOS was mainly expressed in tumor-infiltrating macrophages.

3. Tumor cell proliferation and death. Cell replication and death are the two parameters that determine tumor growth rate. We, therefore, investigated expression of proliferating cell nuclear antigen (PCNA), which is expressed mainly in the late G1 and M phase of the cell cycle and indicates cell replication, by immunohistochemical staining and

apoptosis by TUNEL staining, respectively. On day 7 after the intralesional injection of PBS, H5BVIFN-β, or H5BVIFN-β plus anti-Mac-1 and Mac-2 administration, tumors will be sampled for in vitro analyses. As shown in Fig.2, the majority of cells in tumors injected with PBS were positively stained by antibody to PCNA. The portion of PCNA-positive cells in tumors injected with H5BVIFN-β was significantly reduced (Fig. 2). This effect of H5BVIFN-β was attenuated by administration of Mac-1 and Mac-2 antibodies (Fig.2). No significant apoptosis, revealed by the TUNEL staining, was noted in tumors of any treatment group (Fig. 3). Therefore, retardation of tumor growth by H5BVIFN-β therapy was due mainly suppression of cell proliferation, but not induction of cell death.

4. Effects of H5BVIFN-β therapy on tumor angiogenesis. Since IFN-β is a potent antiangiogenic molecule, we investigated whether H5BVIFN-β therapy altered angiogenesis in the tumor lesions. TRAMP tumors were established and treated with PBS, H5BVIFN-β, or H5BVIFN-β with antibodies against Mac-1 and Mac-2. Tumors were samples on day 3 (not shown) and day 7 and analyzed by immunofluorescent staining using an antibody specific to murine CD31 that is selectively expressed microvessel endothelial cells. As shown in Fig. 3, microvessel density is significantly reduced, approximately by 50%, in tumors injected with H5BVIFN-β. On the other hand, administration of macrophage-specific antibodies partially blocked effects of H5BVIFN-β, suggesting that macrophages were involved in suppression of angiogenesis induced by H5BVIFN-β therapy. The staining of CD31 (microvessel endothelial cells) and TUNEL (apoptotic cells) did not overlap (Fig. 3), suggesting that the reduction of microvessels in tumor lesion by H5BVIFN-β therapy was not due to induction of apoptosis of microvessel endothelial cells.

Next, we analyzed expression of several angiogenic factors in tumor lesions sampled at the same time of those for CD31 staining. Total RNA from the tumors was extracted and analyzed by real-time reverse-transcriptional PCR. As shown in Fig. 4, H5BVIFN-β therapy significantly reduced expression of transforming growth factor (TGF)-β1, one of the most potent angiogenic factor overexpressed in prostate cancer

(Fig. 4A). Similar effects of H5BVIFN-β therapy were observed on expression of endothelin (ET)-1 (Fig. 4B) and to a less extents on vascular endothelial cell growth factor (VEGF) (Fig. 4C) and platelet derived growth factor (PDGF) (Fig. 4D). Administration of the anti-Mac-1 and anti-Mac-2 antibodies attenuated H5BVIFN-β therapy-induced downregulation of the angiogenic molecule expression (Fig. 4D), suggesting the involvement of macrophages in the process, possibly mediated by iNOS.

2. Summary

During the last three years, we investigated therapeutic potential of novel immune modulation system H5BVIFN-β against prostate cancer and the underlying mechanisms of the therapy in a mouse model of prostate cancer. Our data show that H5BVIFN-β therapy was able to partially inhibit growth of orthotopic tumors and prolong the survival of tumor-bearing mice. However, it fails to mount an immune response sufficient to eradicate tumors and protect tumor-bearing mice. The therapeutic effects were much weaker in iNOS-deficient mice and in mice in which macrophages were depleted, suggesting activation of iNOS expression in tumor-infiltrating macrophages is at least one of the mechanisms mediating the therapeutic effects. Intratumoral injection of H5BVIFN-β reduced microvessel density and expression of several angiogenic molecules, implicating that suppression of tumor growth by H5BVIFN-β therapy was possibly mediated by inhibition of tumor angiogenesis.

KEY ACCOMPLISHMENT

1. We have demonstrated, in TRAMP-C2RE3 and TRAMP-L5 tumor models, that intratumoral injection of H5BVIFN-β significantly inhibited orthotopic growth of mouse prostate cancer cells and that the therapy moderately but significantly prolonged the survival of tumor-bearing mice.
2. We showed that unlike in mouse melanoma models, HBVIFN-β therapy could not eradicate orthotopic tumors of mouse prostate cancer or confer immune protection in treated mice.

3. H5BVIFN-β therapy suppressed tumor angiogenesis, which correlated with tumor growth retardation, which was possibly mediated by activation of tumor-infiltrating macrophages.
4. Our data suggest that intratumoral injection of H5BVIFN-β could be a potential novel therapy for human prostate cancer.

REPORTABLE OUTCOME

A manuscript is in preparation for publication.

CONCLUSIONS

1. H5BVIFN-β therapy suppressed tumor growth and prolonged the survival of tumor-bearing mice.
2. H5BVIFN-β suppressed tumor angiogenesis.
3. Tumor-infiltrating macrophages and iNOS expressed in the macrophages play important role in suppressing tumor growth and tumor angiogenesis.
4. H5BVIFN-β may be beneficial to patients with localized prostate cancer.

APPENDICES

FIGURES 1-4.

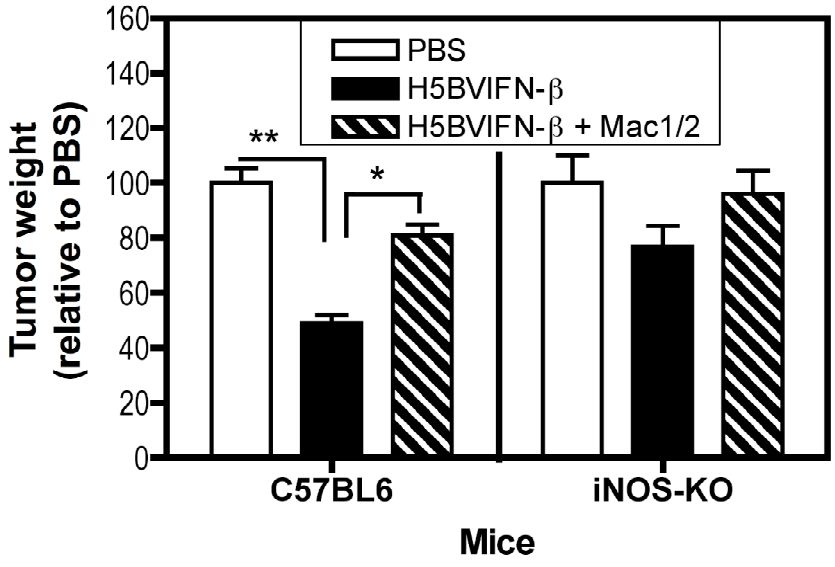
160
140
120
100
80
60
40
20
0
Tumor weight
(relative to PBS)
PBS
H5BVIFN-β
H5BVIFN-β + Mac1/2
**
*
C57BL6
iNOS-KO
Mice

Fig. 1

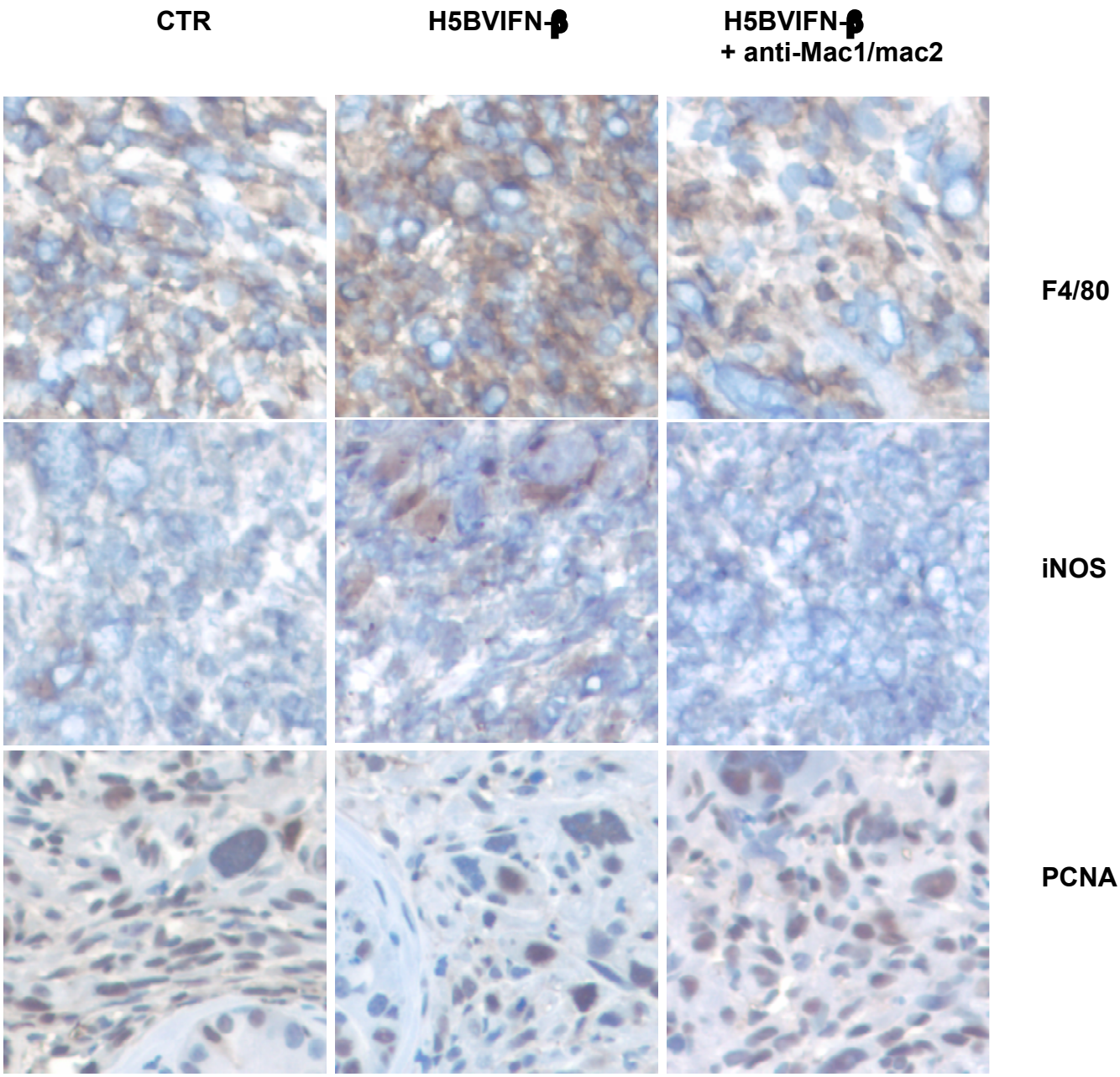

Fig. 2

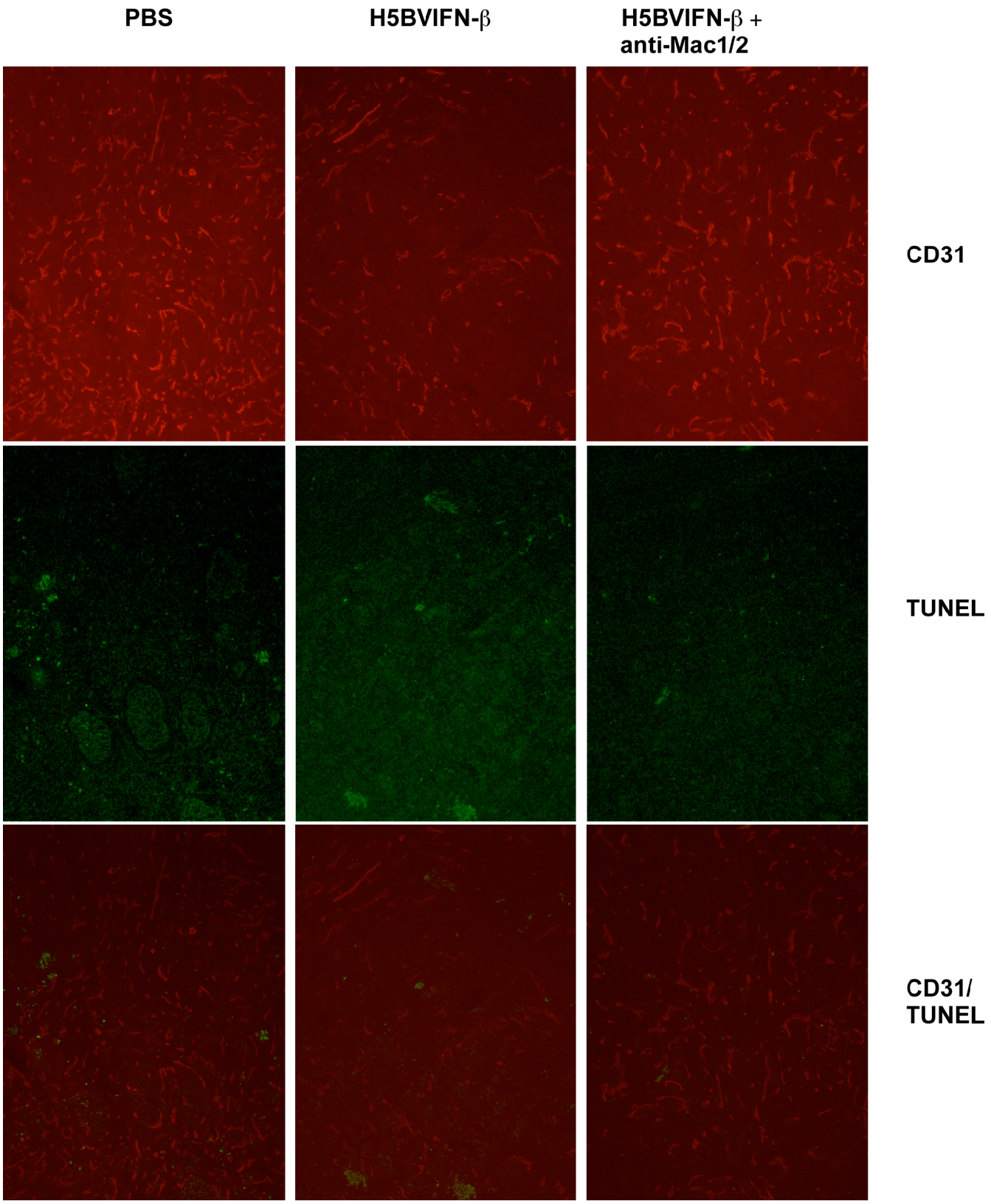

Fig. 3

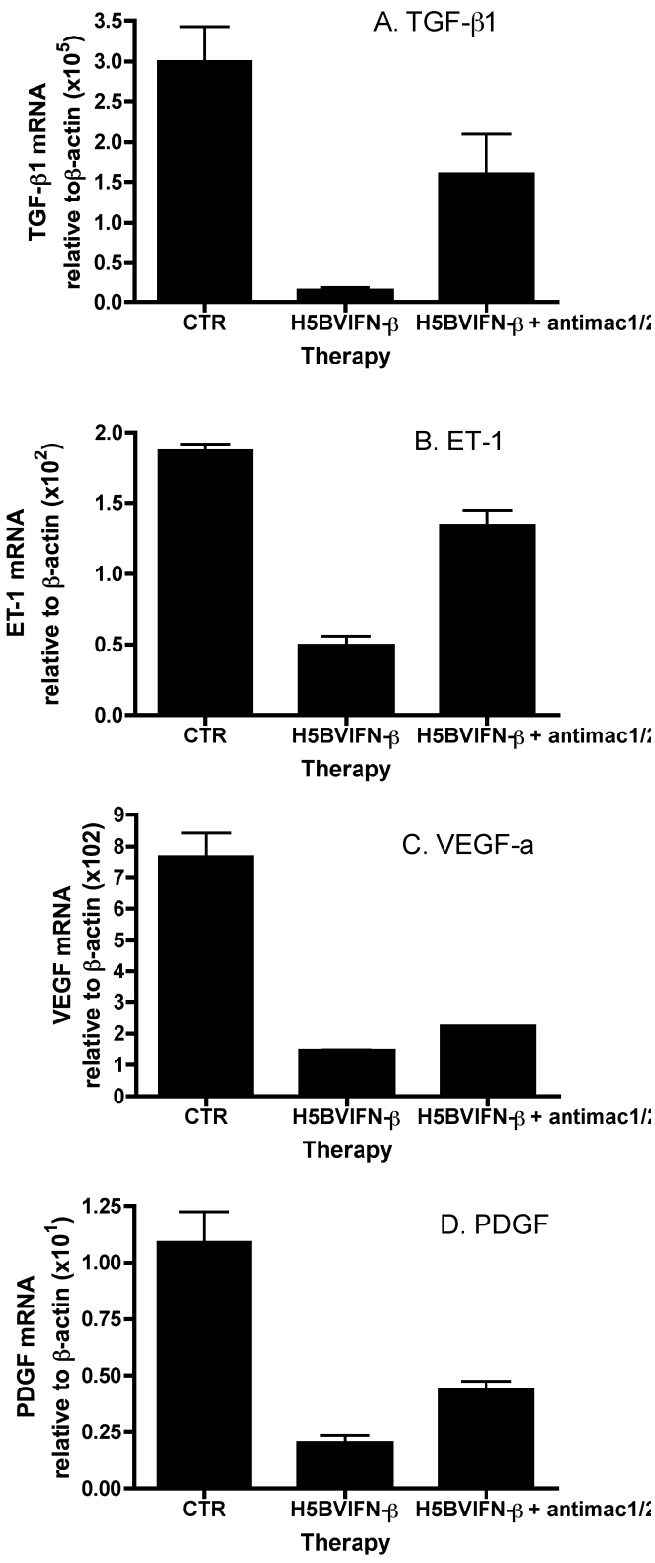
A. TGF-β1
TGF-β1 mRNA relative toβ-actin (x10^5)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CTR
H5BVIFN-β
H5BVIFN-β + antimac1/2
Therapy
B. ET-1
ET-1 mRNA relative to β-actin (x10^2)
2.0
1.5
1.0
0.5
0.0
CTR
H5BVIFN-β
H5BVIFN-β + antimac1/2
Therapy
C. VEGF-a
VEGF mRNA relative to β-actin (x102)
9
8
7
6
5
4
3
2
1
0
CTR
H5BVIFN-β
H5BVIFN-β + antimac1/2
Therapy
D. PDGF
PDGF mRNA relative to β-actin (x10^1)
1.25
1.00
0.75
0.50
0.25
0.00
CTR
H5BVIFN-β
H5BVIFN-β + antimac1/2
Therapy

Fig. 4